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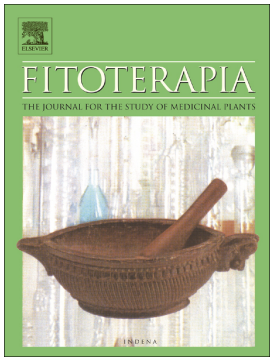

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^{13}C-NMR DEREPLICATION OF GARCINIA EXTRACTS: PREDICTED CHEMICAL SHIFTS AS RELIABLE DATABASES

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Abstract

Usually isolated from *Garcinia* (Clusiaceae) or *Hypericum* (Hypericaceae) species, some Polycyclic Polyprenylated AcylPhloroglucinols (PPAPs) have been recently reported as potential research tools for immunotherapy. Aiming at exploring the chemodiversity of PPAPs amongst *Garcinia* genus, a dereplication process suitable for such natural compounds has been developed. Although less sensitive than mass spectrometry, NMR spectroscopy is perfectly reproducible and allows stereoisomers distinction, justifying the development of ^{13}C-NMR strategies. Dereplication requires the use of databases (DBs). To define if predicted DBs were accurate enough as dereplication tools, experimental and predicted δ_C of natural products usually isolated from Clusiaceae were compared. The ACD/Labs commercial software allowed to predict 73% of δ_C in a 1.25 ppm range around the experimental values. Consequently, with these parameters, the major PPAPs from a *Garcinia bancana* extract were successfully identified using a predicted DB.

Keywords: Clusiaceae; Database; ^{13}C-NMR Dereplication; *Garcinia*; PPAPs; Prediction software

1. Introduction

Manipulating the immune system is a therapeutic approach to either activate immunity against tumors and infected cells or to prevent its activation in autoimmune diseases or transplantation [1, 2]. The Major Histocompatibility Complex (MHC) is a group of genes encoding cell surface proteins that control immune responses in both normal and pathological conditions. In this context, natural products (NPs) able to modulate MHC protein expression may provide new therapeutic strategies for immunotherapy. The authors have recently shown that Polycyclic Polyprenylated AcylPhloroglucinols (PPAPs) such as guttiferone J (**1**) (Figure 1) represent efficient modulators of some MHC proteins [3]. PPAPs share one acylphloroglucinol scaffold, polysubstituted by prenyl or geranyl groups with various degrees of oxidation involved in different types of secondary cyclizations [4]. Guttiferone J (**1**), like many PPAPs, was previously isolated from *Garcinia* species (i.e. *G. virgata* and *G. yunnanensis*) [4-6] which also biosynthesize a variety of different NPs including xanthones, triterpenes, biphenyls, chromanones, coumarins, depsidones and tocotrienols [7]. Exploring the chemodiversity of PPAPs may reveal further valuable leads. However, very few of these PPAPs are commercially available, whereas a wide variety of such derivatives may be isolated from Clusiaceae, Calophyllaceae and Hypericaceae species with 618 entities reported so far [4, 8].

In order to limit time consuming fractionation and purification procedures and rapidly focus on crude extracts containing desired molecular scaffolds, the authors have developed a targeted dereplication protocol specifically adapted for the detection of PPAPs. High-performance liquid chromatography (HPLC) combined with mass spectrometry (MS) is commonly used as method of first choice as it allows to compare retention times, molecular weights and fragmentation patterns with references data [9]. Moreover, these data may be used for building up molecular networks [10]. However, as far as PPAPs are concerned,

asymmetric centers and prenylated/geranylated groups result in fragmentation patterns that are sometimes hardly distinguishable by MS^n [11]. Alternatively, dereplication may also be achieved through nuclear magnetic resonance (NMR): ^{1}H-NMR [12], ^{13}C-NMR [13] or 2D-NMR [14]. As dereplication tools, both MS and NMR exhibit advantages and drawbacks. While MS provides a higher sensitivity, a standard ionization protocol may not be suitable for a wide range of different molecules often present in plant materials. Though direct inspection of polyphenol extracts has proved to be efficient in some specific cases [15, 16], hyphenation with chromatography is generally requested prior to MS analysis. *A contrario*, NMR can be applied to complex mixtures [17]. Moreover, it allows differentiation of stereoisomers, which may be very challenging or even impossible with MS analysis [18]. On the one hand, ^{1}H-NMR spectra are quickly recorded, but ^{1}H chemical shifts (δ_H) are strongly solvent-dependent whereas complex mixtures also generate regions of intense δ_H overlapping which impair spectra interpretation. On the other hand, ^{13}C-NMR requires longer acquisition time, but δ_C are less solvent-sensitive whilst signals overlapping are seldom observed. 2D-NMR may provide additional key information on the spatial structure of the molecule, but for practical reasons these experiments must be recorded with much lower resolution than for 1D-NMR [19]. Therefore, benefitting from the assets of ^{13}C-NMR a dereplication strategy was thus applied in order to quickly identify PPAPs in a *Garcinia bancana* (Clusiaceae) bark extract [20]. In this context, a δ_C database (DB) was required.

The literature suggests that several types of DBs can be used. First, the use of an internal DB gathering the experimental chemical shifts of secondary metabolites isolated and analysed by a given laboratory is possible. In such a case, it is possible to identify compounds with an accuracy of 0.1 ppm if all analyses are conducted in the same deuterated solvent and with the same parameters [17]. Then, working on more complex mixtures, it is usually impossible to use the same NMR conditions, or to have an extensive internal DB at our disposal. Therefore,

^{13}C chemical shifts can be gathered from the literature to build a bigger "experimental DB" [21]. However, this type of DB is quite long to build and obtaining an exhaustive one is a difficult task. For the present work, this is even more difficult with PPAPs for which NMR data are usually reported in pyridine-d6, deuterated chloroform and methanol-d4 with or without 0.1% TFA, generating strongly solvent dependant DBs. The third and last option is the construction of a theoretical DB using a ^{13}C-NMR prediction software. To our knowledge, reported ^{13}C-NMR dereplication processes indifferently rely on experimental or predicted DBs but, without real justification [22]. Hence, this work will try to figure out if predictive DBs are accurate enough to be used as a general dereplication tool suitable for *Garcinia* extracts.

2. Experimental

2.1. Predictive and experimental databases

As a general validation trial, 80 NPs representative of the Clusiaceae family, chosen for their structural diversity and including benzophenones, biphenyls, chromanones, coumarins, depsidones, PPAPs, tocotrienols, triterpenes and xanthones (Table S1), were included in both an experimental DB1 and a predictive DB2. For the experimental one, structure-data files (SDF) of these metabolites were built using *ChemSketch* application (ACD/Labs Release 12.00) whilst literature chemical shifts were entered into a Microsoft Excel 2016 spreadsheet. For the predictive DB, SDF were analyzed with three different software programs: *Spectrus Processor* from ACD/Labs (2014 release), the module NMRP from MestReNova (v12.0.1) and ChemDraw Professional (v 17.0.0.206). The absolute difference between experimental and predicted ^{13}C-NMR chemical shifts was calculated for each NP. The PivotTable feature allowed the evaluation of the distance between predicted and experimental values, ranked by accuracy, depending on the type of structure or carbon type, making the interpretation easier.

2.2. Method validation on a Garcinia bancana extract

2.2.1. Plant material

Garcinia bancana bark was collected in October 2000 around Mersing, Johor. The plant was identified by the botanist Mr. Teo Leong Eng and the voucher specimen (KL4967) was deposited at the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

2.2.2. Fractionation

10 g of bark powder were successively extracted by sonication (3 h) with dichloromethane (740 mg) and methanol (1.35 g). 650 mg of the dichloromethanic extract were then fractionated using normal phase flash chromatography on silica gel (Chromabond® flash RS 40 SiOH) from 100 % cyclohexane to 70% cyclohexane / 30% ethyl acetate (flow: 20 mL/min). 10 successive sub-fractions likely to contain PPAPs (LC-UV, ^{1}H-NMR) were selected for ^{13}C-NMR dereplication: F1 (15.2 mg), F2 (12.2 mg), F3 (13.2 mg), F4 (18.8 mg), F5 (30.1 mg), F6 (6.5 mg), F7 (53.7 mg), F8 (27.6 mg), F9 (22.9 mg) and F10 (25.9 mg).

2.2.3. NMR experiments

^{13}C-NMR experiments of the fractions and pure products were done in deuterated methanol and 0.1% trifluoroacetic acid using the JEOL 400 MHz YH spectrometer (Jeol Europe). Parameters were 16000 scans for 15 mg of fraction.

2.2.4. Bucketing and cluster analysis

A bucketing step was automated using a macro on a Microsoft Excel spreadsheet. The hierarchical cluster analysis (HCA), allowing the formation of chemical shifts clusters, was

processed using Permutmatrix 1.9.3 software [23]. HCA can recognize and sort specific set of ^{13}C-NMR chemical shifts, each set is supposed to belong to a different molecule.

2.2.5. Compounds purification and identification

30 mg of fraction 7 were separated using semi-preparative HPLC (Agilent HP 1100 Series, Agilent Technologies, Les Ulis, France) on a reversed phase column (Phenomenex Luna C18, 100 Å, 250 x 10 mm, 5 µm), using a 50 mg/mL concentration for the injection (100 µL), with a 97% methanol + 0.1% formic acid / 3 % water + 0.1% formic acid system (flow: 2.8 mL/min). Fractions were collected using the Agilent Technologies 1260 Infinity G1364C fraction collector and the ChemStation for LC 3D software for automatic UV peak detection (diode array detector G13115A). This led to 8.7 mg of xanthochymol (**2**) [24] and guttiferone F (**3**) [25] as a mixture.

Another semi-preparative HPLC on a different column (Hypersil Gold PFP, 150 mm x 10 mm), using a 50 mg/mL concentration for the injection (100 µL), with a 75% methanol / 25% water + 0.1% formic acid system (flow: 4.7 mL/min) yielded 2.5 mg of xanthochymol (**2**) and 1.5 mg of guttiferone F (**3**) from fraction 6. The same method led to the purification of 2.9 mg of xanthochymol (**2**), 3.6 mg of guttiferone F (**3**) and 4.3 mg of 30-epi-cambogin (**4**) [25] from fraction 7 (Figure 1).

2.2.6. Database choice for G. bancana

A predictive DB3 in *C+H NMR Predictor* (ACD/Labs) was built using a SDF gathering 718 NPs described in the *Garcinia* genus on the Dictionary of Natural Products website [26]. On the other hand, the CH-NMR-NP JEOL database [27] was used as the experimental DB4. This website gathers the ^{13}C-NMR chemical shifts of around 30,500 NPs published between 2000 and 2014, including compounds from *Garcinia*.

3. Results and discussion

3.1. Accuracy of the chemical shift prediction (DB1 vs DB2)

In average, as far as 80 NPs isolated from Clusiaceae and Calophyllaceae species were concerned, the ACD/Labs software could predict 73% of carbon chemical shifts with a ± 1.25 ppm accuracy versus 2.00 and 3.00 ppm for MestRecNova and ChemDraw software programs respectively (Table 1). It also appeared that the accuracy of these prediction software strongly depended on NPs structural class but not on the type of carbon hybridization.

3.2. Dereplication analysis on Garcinia bancana extract

Garcinia genus is known for producing various PPAPs of biological interest but also different kind of secondary metabolites [4]. To focus on PPAPs, a preliminary study was conducted on 124 DCM and MeOH extracts from the bark, leaf, and sometimes fruit of 17 Malaysian *Garcinia* species (30 batches): LC-UV-MS^2 analyses suggested PPAPs as major compounds in 19 DCM extracts including in a *G. bancana* bark extract. However, their structures could not be firmly characterized using MS^2 data due to the similar fragmentation pattern of the stereoisomers. Little work has been reported on this endemic Malaysian plant. Only two PPAPs (*i.e.* garcinol and isogarcinol), two isocoumarin derivatives, two triterpenes, a monoterpene glycoside and a biphenyl were isolated from the twigs when two flavones glycosides and tannins were reported in the leaves [28-30]. Thus, focusing on PPAPs, an NMR dereplication study was conducted on this DCM bark extract, requiring a database of NPs and their chemical shifts.

Exploring the potential of predicted DBs, we turned to a ^{13}C-NMR dereplication using a fractionation step, as nicely described by Hubert *et al.* [20]. The hierarchical cluster analysis (HCA) step established 4 clusters corresponding to the major NPs across the several fractions. For each cluster, the set of chemical shifts were searched both in the predicted DB3 (*Garcinia*'s DNP) and DB4 (JEOL). The parameters defined in the previous experiment were

used as query parameters (*i.e.* looseness factor 1.25 ppm; 75% of carbon signals matching). As shown in Figure 2, both DBs managed to identify similar structural type of PPAPs but DB3 and DB4 often predicted different isomers. Thus, major NPs, namely xanthochymol (**2**), guttiferone F (**3**) and 30-epi-cambogin (**4**) (MSI level 1), were purified to determinate if the different DBs properly identified them. Predicted DB3 correctly suggested xanthochymol, stereoisomers of guttiferone F (garcinol or guttiferone E) and one isomer of 30-epi-cambogin (cyloxanthochymol). Concerning the experimental DB4, it proposed one isomer of guttiferone F (garcinielliptone FC) and one stereoisomer of 30-epi-cambogin (isogarcinol). Actually, as the experimental JEOL DB4 did not include the NPs of the crude extract, a perfect match was not possible.

4. Conclusion

To explore efficiently the chemodiversity of PPAPs and their ability to modulate MHC molecules expression, we have been developing a dereplication process suitable for *Garcinia* species. It should be noticed that LC-MS² analyses could not distinguish the different PPAPs stereoisomers. Therefore, a ^{13}C-NMR dereplication study was chosen requiring an appropriate DB. When available, working with an experimental DB is ideal, increasing the chance for better matches if the NMR experiments are conducted in the same solvent. However, as no experimental DBs are exhaustive and require a long time to build, this work demonstrates for the first time that predictive DBs are an attractive alternative, at least for first dereplication steps. Indeed, the accuracy (± 1.25 ppm for 75% δ_C) of one predicted DB3 was sufficient to identify the major PPAPs successively isolated from a *Garcinia bancana* crude extract. As far as Clusiaceae and Calophyllaceae NPs are concerned, this work also shows great differences in prediction accuracies depending on software programs.

Appendix A. Supplementary data

CAS number, name and structural class of all molecules used to compare the difference between their experimental chemical shifts and the chemical shifts that were predicted by a software for these same structures (Table S1); CAS numbers of PPAPs dereplicated and then isolated from *Garcinia bancana* bark (Table S2).

Appendix B. Supplementary data

The DB3 containing the predicted chemical shifts [C+H NMR Predictor (ACD/Labs)] of 718 compounds described in *Garcinia* species (Dictionary of Natural Products) is available as a SDF: Garcinia DB ^{13}C NMR Chemical shifts.sdf

Conflict of interest

K. Awang, A Bruguière, C. Coste, S. Derbré, M. Le Bot, S. T. Leong, P. Richomme, B. Siegler and S. N. Sulaiman declare no conflict of interest.

Author contributions

This article is a part of the results obtained by AB during his doctoral thesis. STL and SNS, supervised by KA, extracted and fractionated *Garcinia bancana* bark extract. CC was involved in PPAPs purification. MLB and BS automated the bucketing step using a macro on a Microsoft Excel spreadsheet. This work was supervised by SD and PR. AB prepared the figures and tables. AB, SD and PR wrote the manuscript together. All authors discussed the results from the experiments and commented on the manuscript.

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Tables and figures captions

Table 1. Percentage of carbon signals predicted in a given interval using ACD/Labs prediction (blue), MestReNova (Orange) or ChemDraw (green) software. For each prediction software and each structural class, the percentage of carbon signals that have their predicted chemical shifts in a given interval (in ppm) around the experimental chemical shift value is shown. For example, concerning the ACD/Labs prediction software, in average, 73% of the predicted carbon signals are less than 1.25 ppm away from the value described in the literature.

Figure. 1 Structures of guttiferone J (1) and of the compounds isolated from *Garcinia bancana* bark: xanthochymol (2), guttiferone F (3) and 30-epi-cambogin (4).

Figure 2 Molecules predicted for each cluster by the experimental DB (on the left) and the predicted DB (on the right). After hierarchical cluster analysis (HCA) of the fractions, this map was produced, showing the presence (green square) or absence (black square) of a given chemical shifts in one of the fractions. Clusters of green squares are recognized as chemical shifts belonging to the same molecule. Those chemical shifts were thus entered as query in both DBs. For each cluster and for each DB, the structure of the first proposed molecule is displayed. The predicted DB also shows its ranking. In cluster A for example, the software proposed 7 molecules as a potential match (out of the 718 in this DB), 13, 14-dedioxyisogarcinol being the one with the higher score.

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Table 1

ACD/Labs

% of carbon signals	Structural class									Average
Absolute difference	Benzophenone	Biphenyl	Chromanone	Coumarin	Depsidone	PPAP	Tocotrienol	Triterpene	Xanthone	
ppm < 1.00	41%	60%	66%	65%	65%	67%	90%	80%	64%	68%
ppm < 1.25	53%	67%	72%	69%	68%	72%	92%	86%	70%	73%
ppm < 1.50	63%	77%	74%	73%	68%	77%	94%	89%	75%	77%
ppm < 1.75	69%	77%	76%	75%	69%	80%	95%	90%	79%	79%
ppm < 2.00	69%	80%	80%	78%	69%	82%	96%	92%	81%	81%
ppm < 3.00	79%	85%	86%	87%	72%	89%	97%	94%	88%	87%
ppm < 4.00	86%	92%	92%	90%	75%	95%	99%	97%	93%	92%
ppm < 5.00	88%	96%	94%	93%	85%	98%	100%	97%	94%	95%
ppm < 10.00	98%	100%	98%	99%	99%	100%	100%	99%	98%	99%
ppm < 20.00	100%	100%	99%	100%	100%	100%	100%	100%	99%	100%
20.00 ≤ ppm	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

MNova

% of carbon signals	Structural class									Average
Absolute difference	Benzophenone	Biphenyl	Chromanone	Coumarin	Depsidone	PPAP	Tocotrienol	Triterpene	Xanthone	
ppm < 1.00	38%	35%	53%	40%	28%	63%	56%	74%	49%	51%
ppm < 1.25	46%	43%	65%	47%	35%	69%	68%	77%	57%	58%
ppm < 1.50	49%	49%	69%	53%	38%	74%	74%	79%	59%	63%
ppm < 1.75	56%	57%	73%	58%	43%	78%	81%	83%	64%	68%
ppm < 2.00	60%	61%	75%	61%	46%	80%	84%	87%	66%	70%
ppm < 3.00	63%	73%	85%	72%	54%	86%	87%	91%	78%	79%
ppm < 4.00	75%	84%	91%	84%	66%	92%	93%	94%	89%	87%
ppm < 5.00	84%	92%	94%	86%	83%	96%	94%	97%	93%	92%
ppm < 10.00	99%	99%	99%	95%	98%	100%	99%	99%	98%	98%
ppm < 20.00	100%	100%	100%	99%	100%	100%	100%	100%	99%	100%
20.00 ≤ ppm	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

ChemDraw

% of carbon signals	Structural class									Average
Absolute difference	Benzophenone	Biphenyl	Chromanone	Coumarin	Depsidone	PPAP	Tocotrienol	Triterpene	Xanthone	
ppm < 1.00	38%	37%	39%	38%	29%	42%	69%	76%	43%	45%
ppm < 1.25	48%	50%	46%	51%	36%	51%	78%	81%	51%	54%
ppm < 1.50	55%	57%	49%	58%	38%	58%	81%	85%	56%	59%
ppm < 1.75	59%	61%	53%	63%	40%	65%	84%	85%	62%	63%
ppm < 2.00	60%	63%	54%	64%	44%	68%	87%	90%	65%	66%
ppm < 3.00	70%	87%	68%	72%	51%	81%	92%	96%	73%	76%
ppm < 4.00	75%	91%	74%	76%	64%	88%	94%	97%	81%	82%
ppm < 5.00	77%	91%	81%	81%	73%	93%	95%	97%	86%	87%
ppm < 10.00	94%	99%	94%	95%	96%	97%	100%	99%	95%	97%
ppm < 20.00	100%	100%	100%	98%	100%	100%	100%	100%	98%	99%
20.00 ≤ ppm	100%	100%	100%	100%	100%	100%	100%	100%	100%	100%

Figure 1

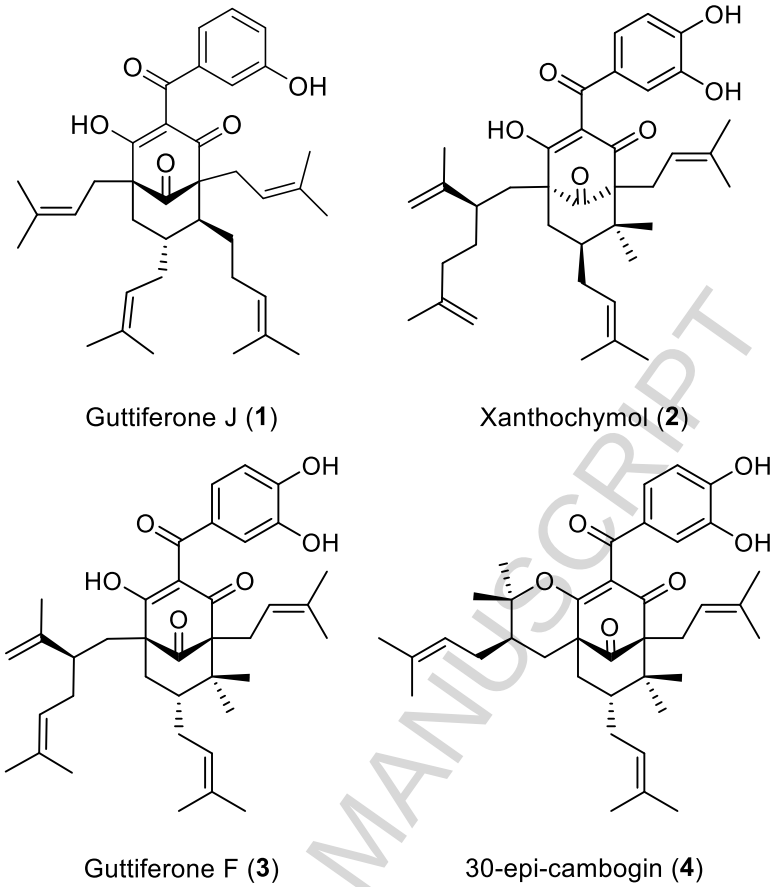

Figure 2